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Identification of *Aedes aegypti* and Its Respective Life Stages by Real-Time Polymerase Chain Reaction

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An *Aedes aegypti*-specific, fluorogenic probe hydrolysis (TaqMan), polymerase chain reaction assay was developed for real-time screening using a field-deployable thermocycler. Laboratory-based testing of *A. aegypti*, *A. aegypti* (Trinidad strain), *Culex pipiens*, *Culex quinquefasciatus*, *Anopheles stephensi*, and *Ochlerotatus taeniorhynchus* individual adult mosquitoes and mixed pools (n = 10) demonstrated 100% concordance in both in vitro sensitivity (six of six samples) and specificity (10 of 10 samples). A single adult *A. aegypti* was identified in a pool of 100 non-*A. aegypti* mosquitoes. The limit of detection of *A. aegypti* egg pools was five individual eggs. Field testing was conducted in central Honduras. An *A. aegypti* and *Culex* spp. panel of individual and mixed pools (n = 30) of adult mosquitoes, pupae, and larvae demonstrated 100% concordance in sensitivity (22 of 22 samples) and 97% concordance in specificity (29 of 30 samples), with one false-positive result. Field testing of an *A. aegypti* and *Culex* spp. blind panel (n = 16) consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae demonstrated 90% concordance in sensitivity (nine of 10 samples) and 88% concordance in specificity (14 of 16 samples).

Introduction

The anticipation, prediction, identification, prevention, and control of vector-borne disease threats to military personnel are critical in all military operations. Real-time surveillance of mosquitoes and their respective immature stages allows rapid assessment of potential disease transmission risk and timely implementation of appropriate control measures. *Aedes aegypti* is the primary vector of dengue fever and yellow fever viruses, thus representing a substantial threat for disease transmission to humans in many subtropical and tropical regions of the world.[1] Dengue fever is the most significant mosquito-borne viral disease today, with a risk comparable to that for malaria, i.e., two-fifths of the world's human population.[2,3] Although malarial disease can be prevented by prophylaxis and yellow fever by immunization, dengue fever prophylaxis does not exist and an approved vaccine is not anticipated in the near future. Currently, the only method of preventing infection with the dengue virus is vector avoidance.

A. aegypti is a peridomestic, diurnally active mosquito that prefers to breed in artificial containers near human habitations. Transmission of viruses to humans is by blood-feeding female mosquitoes exclusively, because male mosquitoes do not bite. Vertical and possibly venereal transmission of dengue virus occurs from infected female mosquitoes to their progeny (transovarian)[4,5] and from infected male mosquitoes to female mosquitoes during copulation, respectively.[6] Therefore, although male mosquitoes do not directly infect humans, they must be considered in the transmission cycle. In the absence of viremic hosts, these modes of transmission ensure survival of viruses in nature.

Control of disease transmission in disease-endemic regions has become progressively more challenging as container-breeding mosquito habitat increases with exponentially increasing human populations and diminishing public resources for planning and controlling urban development.[1] Depletion of public health resources has resulted in a lack of, or inefficient, mosquito control. Expanding global travel has exacerbated the problem by driving virus circulation in previously non-disease-endemic regions, thereby enhancing the potential for epidemics. Moreover, global warming influences local climatic patterns, potentially making them more favorable for establishment and development of *A. aegypti*.[7-9]

Rapid identification of both pathogens and their arthropod vectors is paramount for protecting military personnel.[10] Similarly, surveillance of mosquitoes and their respective immature stages allows continued assessment of potential transmission risk and timely implementation of appropriate mosquito control measures. However, many military entomologists lack the taxonomic skills necessary to accurately identify vectors beyond the genus level. Public health personnel who are often tasked with conducting entomological surveillance generally are less experienced in species identification. In the U.S. Air Force, ar-

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14. ABSTRACT

An Aedes aegypti-specific, fluorogenic probe hydrolysis (Taq-Man), polymerase chain reaction assay was developed for real-time screening using a field-deployable thermocycler. Laboratory-based testing of A. aegypti, A. aegypti (Trinidad strain), Culex pipiens, Culex quinquefasciatus, Anopheles stephensi, and Ochlerotatus taeniorhynchus individual adult mosquitoes and mixed pools (n = 10) demonstrated 100% concordance in both in vitro sensitivity (six of six samples) and specificity (10 of 10 samples). A single adult A. aegypti was identified in a pool of 100 non-A. aegypti mosquitoes. The limit of detection of A. aegypti egg pools was five individual eggs. Field testing was conducted in central Honduras. An A. aegypti and Culex spp. panel of individual and mixed pools (n = 30) of adult mosquitoes, pupae, and larvae demonstrated 100% concordance in sensitivity (22 of 22 samples) and 97% concordance in specificity (29 of 30 samples), with one false-positive result. Field testing of an A. aegypti and Culex spp. blind panel (n = 16) consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae demonstrated 90% concordance in sensitivity (nine of 10 samples) and 88% concordance in specificity (14 of 16 samples).

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thropods (primarily mosquitoes and ticks) collected during routine surveillance are packaged and shipped to an out-of-area laboratory for identification by an entomologist with taxonomic skills. Although this approach is largely successful for obtaining specific identifications of potential vectors, the time involved in this process often conflicts with the requirement for rapid specific identification to help in the prediction and prevention of vector-borne disease outbreaks. For example, the U.S. Air Force primarily uses ovitraps to conduct base-level surveillance for *Aedes (Stegomyia)* mosquitoes and then rears the collected eggs to obtain adults for positive identification.[11–15] However, under field conditions, especially in areas where disease transmission is active or where environmental conditions prohibit the use of ovitraps,[16] this method may not be practical. Identification of *Aedes (Stegomyia)* mosquitoes under field conditions also may not be practical when adults are not present, and identification of immature stages can prove challenging for untrained personnel. Moreover, there are occasionally requirements to conduct mosquito surveillance over a large geographical area or from a large number of locations, which may involve the separation of the immature stages of *A. aegypti* and related species and/or the laboratory rearing of mosquitoes from positive ovitraps.[17] Because of space and time requirements, substantial logistical problems can arise in such large-scale studies.[18]

Efficacious surveillance of vector species, and their pathogens, is fundamental to the assessment of disease risk and the time-critical implementation of appropriate transmission prevention measures and mosquito control. We describe here a real-time polymerase chain reaction (PCR) assay for sensitive specific identification of *A. aegypti* and its respective life stages, using field-deployable instrumentation.

Methods

Primer and Probe Design

Optimal probe and primer sequences were computed using Primer Express software according to the manufacturer's instructions (PE Applied Biosystems, Foster City, California). Primer sequences were identified with a (Tm) (melting temperature) of 10°C less than the Tm of the probe. The fluorescent reporter molecule at the 5′ end of the TaqMan probe (Synthetic Genetics, Rockville, Maryland) was 6-carboxyfluorescein and the quenching molecule was 6-carboxytetramethyl-rhodamine. Primers and probe oligonucleotides were synthesized commercially (Synthetic Genetics, Rockville, Maryland). Requests for sequences can be submitted through the corresponding author.

Assay Optimizations

Preliminary assay optimization was performed with a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) and transferred to the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) (Idaho Technology, Salt Lake City, Utah) using fluorogenic probe hydrolysis (TaqMan)-based PCR.[19,20] Graphics of the R.A.P.I.D. can be found at the Idaho Technology World Wide Web site (www.idahotech.com). Assays were optimized with a proprietary buffer system (Idaho Technology), and sensitivity and specificity validation testing was completed.

Reaction Conditions

Assay optimizations and cross-reaction testing were conducted on the R.A.P.I.D. before sensitivity and specificity validation testing. Master mixture solution was prepared, 18-μL volumes were dispensed into optical capillary tubes, and 2 μL of DNA extract from specimens and control samples were added (or 2 μL of PCR-grade water for no-template control samples). Capillaries were placed in a tabletop centrifuge and centrifuged for 2 to 3 seconds at 3,000 rpm, to drive the assay mixture to the bottom of the tube. Master mixture components were 2× Quantitech probe PCR master mix (Qiagen, Valencia, California). The forward primer concentration was 0.30 μM, reverse primer 0.90 μM, and TaqMan probe 0.10 μM. A standardized reverse transcription-PCR thermal cycling protocol was established, consisting of initial DNA denaturation at 94°C for 2 minutes and PCR for 45 cycles of 94°C for 0 seconds for template denaturation and 60°C for 20 seconds for combined annealing and primer extension. A single data point at the end of each annealing-extension cycle was collected and reported as TaqMan probe fluorescence released by 5′-nuclease activity during primer extension. Fluorimeter gains were set at 8, 2, and 2 for channels 1, 2, and 3, respectively. The criterion for a positive result was a significant increase in fluorescence over background levels, i.e., threshold PCR cycle (Ct), as defined by an algorithm provided in the R.A.P.I.D analytical software (Roche Molecular Biochemicals, Indianapolis, Indiana).

Laboratory Evaluations of *A. aegypti* PCR Assays

Mosquito Panels

Evaluations of the *Aedes* genetic assay for sensitivity and specificity were accomplished under controlled conditions at Air Force Air Institute for Environment, Safety, and Occupational Health Risk Analysis (AFIERA). Laboratory evaluations were conducted with adult mosquitoes (*A. aegypti, Anopheles stephensi, Culex pipiens, Culex quinquefasciatus*, and *Ochlerotatus taeniorhynchus*), various pools of these species, and *A. aegypti* eggs provided by the Department of Virology, U.S. Army Research Institute of Infectious Diseases (Fort Detrick, Maryland) (Tables I, II, and III). Species identification and confirmation were accomplished with morphological examinations and serological analyses by U.S. Army entomologists. Mosquitoes were held in cardboard cages, provided with a carbohydrate source (either apple slices or a gauze pad soaked in a 10% sucrose solution) and a water-soaked cotton pledget, and held at 26°C for 7 days. Mosquitoes were then killed by exposure to −20°C for 5 to 10 minutes, placed in sterile, 1.5-mL, Eppendorf tubes, and triturated in 750 μL of TRIzol-LS (Life Technologies, Rockville, Maryland). Panels established at the U.S. Army Research Institute of Infectious Diseases were shipped on dry ice to Molecular Epidemiology, AFIERA, Brooks Air Force Base (San Antonio, Texas), for nucleic acid extraction and PCR analyses.

DNA Preparation

Single adult mosquitoes and mosquito pools were placed in sterile, 1.5-mL, Eppendorf tubes and homogenized with a sterile, blunted, 1,000-μL pipette tip in 200 μL of sterile water. Sample homogenates were centrifuged for 60 seconds at 13,500 rpm on a tabletop centrifuge, and ~200 μL of supernatant were

TABLE I

LABORATORY EVALUATION OF *Aedes aegypti* PCR ASSAY SENSITIVITY AND SPECIFICITY

Sample	Sample Composition	n^a	PCR Results	Cycles (C_t)
Sample				
1.1A	*A. aegypti*	1	Positive	20.15
1.1B	*A. aegypti*	1	Positive	20.88
2.1A	*Ochlerotatus taeniorhynchus*	1	Negative	
2.1B	*O. taeniorhynchus*	1	Negative	
3.1A	*Culex pipiens*	2	Negative	
3.1B	*C. pipiens*	2	Negative	
4.1A	*A. aegypti* (Trinidad strain)	2	Positive	26.93
4.1B	*A. aegypti* (Trinidad strain)	2	Positive	26.39
5.1A	*C. pipiens quinquefasciatus*	2	Negative	
5.1B	*C. pipiens quinquefasciatus*	2	Negative	
6.1A	*Anopheles stephensi*	2	Negative	
6.1B	*A. stephensi*	2	Negative	
Mosquito pools (*A. aegypti*/ non-*A. aegypti*)				
7.1A	*A. aegypti*/*A. aegypti* (Trinidad strain)	1/24	Positive	25.73
7.1B	*A. aegypti*/*A. aegypti* (Trinidad strain)	1/24	Positive	26.43
8.1A	*A. aegypti*/*C. pipiens*	1/24	Positive	34.18
8.1B	*A. aegypti*/*C. pipiens*	1/24	Positive	33.61
9.1A	*A. aegypti*/*C. pipiens quinquefasciatus*	1/24	Positive	33.95
9.1B	*A. aegypti*/*C. pipiens quinquefasciatus*	1/24	Positive	34.52
10.1A	*A. aegypti*/*O. taeniorhynchus*	1/24	Positive	32.37
10.1B	*A. aegypti*/*O. taeniorhynchus*	1/24	Positive	32.29

[a]*n* = number of mosquitoes.

TABLE II

LABORATORY EVALUATION OF *Aedes aegypti* PCR ASSAY LIMIT OF DETECTION IN MOSQUITO POOLS

Sample	Sample Composition	n^a	PCR Results	Cycles (C_t)
1.2A	*A. aegypti*/non-*A. aegypti*	1/50	Positive	28.94
1.2B	*A. aegypti*/non-*A. aegypti*	1/50	Positive	28.72
1.2C	*A. aegypti*/non-*A. aegypti*	1/50	Positive	30.96
2.2A	*A. aegypti*/non-*A. aegypti*	1/75	Positive	33.64
2.2B	*A. aegypti*/non-*A. aegypti*	1/75	Positive	33.21
3.2A	*A. aegypti*/non-*A. aegypti*	1/100	Positive	33.51
3.2B	*A. aegypti*/non-*A. aegypti*	1/100	Positive	33.91

[a]*n* = number of mosquitoes.

TABLE III

LABORATORY EVALUATION OF *Aedes aegypti* PCR ASSAY LIMIT OF DETECTION

Sample	Sample Composition	n^a	PCR Results	Cycles (C_t)
1.3	*A. aegypti* eggs	100	Positive	30.15
2.3	*A. aegypti* eggs	50	Positive	32.96
3.3	*A. aegypti* eggs	10	Positive	34.7
4.3	*A. aegypti* eggs	5	Positive	30.68
5.3	*A. aegypti* eggs	1	Negative	

[a]*n* = number of mosquito eggs.

pipetted into the MagNAPure LC sample cartridge for processing. Nucleic acid was isolated using the MagNAPure LC system and MagNAPure LC total nucleic acid isolation kit (Roche Diagnostics, Mannheim, Germany).[21,22] All postloading processing was completed in a closed system by automated robotics, with preformatted reagents and a nucleic acid isolation matrix. Cell lysis and nucleic acid stabilization were completed with buffer containing guanidinium thiocyanate and proteinase K. Nucleic acid bound to the surface of magnetic glass particles was isolated from other cellular components by washing and eluting with a low-salt buffer. Nucleic acid extraction of mosquito eggs was with TRIzol (Life Technologies) according to the manufacturer's instructions, with the exception that sample homogenate was centrifuged for 60 seconds at 13,500 rpm on a tabletop centrifuge and 500 μL of supernatant were exposed to the extraction process.

Field Evaluations of *A. aegypti* PCR Assays

A dengue fever-endemic region (central Honduras) was chosen as the field site, September 17 to 25, 2002.[7] Primary sampling was conducted in Comayagua and Tegucigalpa. Two teams of approximately three or four people each, consisting of entomologists, physicians, public health professionals, and technicians, used battery-powered, hand-held aspirators to collect mosquitoes from the homes of consenting individuals, discarded tires, and other structures. Immature mosquitoes were collected from various natural and man-made containers when present. For the field evaluations, all life stages, exclusive of eggs, were evaluated. Graphics of adult, larval, pupal, and egg stages can be found at the University of Florida, Institute of Food and Agricultural Sciences, World Wide Web site (http://edis.ifas.ufl.edu/IN473).

Captured adult mosquitoes were temporarily (≤3 hours) held in storage tubes placed on dry ice, and immature stages were held in "mosquito breeders" and returned to the field laboratory for processing. Additional specimens of pupae and larvae were collected and preserved in 95% ethyl alcohol for later identification and verification. U.S. Air Force entomologists identified and pooled captured and reared live adult mosquitoes, pupae, and larvae. Specimens were pooled as *A. aegypti* alone and in various combinations with *Culex* spp. Adult mosquitoes were placed in a freezer until they were rendered moribund, they were immediately transferred into 500 μL of Trizol, and then nucleic acids were extracted as described above. Larvae and pupae were placed directly into Trizol reagent before the extraction process. Optimized PCR assays (as described above) were conducted using the R.A.P.I.D.

Two experiments were conducted on the field-collected mosquitoes. In the first experiment, the R.A.P.I.D. operator had previous knowledge of the species composition in each prepared pool (Table IV). In the second experiment, the operator was provided mosquito pools as blind samples of unknown identity and composition (Table V).

Results

Laboratory Evaluations

Sensitivity and specificity testing in laboratory evaluations showed the assay to be highly efficacious, with excellent levels of detection for this species. Laboratory testing of individual adult mosquitoes and mixed mosquito pools demonstrated 100% concordance in both in vitro sensitivity (six of six samples) and specificity (10 of 10 samples) testing. Six doublet sets of *A. aegypti*-positive samples were correctly identified in a panel of 10 doublet sets of *A. aegypti* and non-*A. aegypti* mixed samples, with no cross-reactivity (Table I). Single adult *A. aegypti* were identified in pools of 100 non-*A. aegypti* mosquitoes (Table II), and the limit of detection of *A. aegypti* egg pools was five eggs (Table III). Pools of >100 were not evaluated in this study, and

TABLE IV

PRELIMINARY FIELD EVALUATION OF *Aedes aegypti* PCR ASSAY SENSITIVITY AND SPECIFICITY

Sample	Sample Composition: Known Panel	*n*[a]	PCR Results	Cycles (C_t)
1.4	*A. aegypti* female	1	Positive	32.8
2.4	*A. aegypti* female	1	Positive	32.7
3.4	*Culex* female	1	Negative	
4.4	*Culex* female	1	Positive	40.52
5.4	*A. aegypti* female	2	Positive	29.88
6.4	*A. aegypti* female	2	Positive	34.72
7.4	*A. aegypti* male	2	Positive	32.05
8.4	*A. aegypti* male and female	15	Positive	29.7
9.4	*A. aegypti* female/*Culex* male and female	1/12	Positive	30.91
10.4	*A. aegypti* female/*Culex* male and female	1/12	Positive	32.48
11.4	*A. aegypti*	1	Positive	28.37
12.4	*A. aegypti*	1	Positive	28.97
13.4	*A. aegypti* larva	1	Positive	26.01
14.4	*A. aegypti* larva	1	Positive	27.31
15.4	*A. aegypti* larva	1	Positive	26.57
16.4	*A. aegypti* larva	1	Positive	25.97
17.4	*A. aegypti* pupa	1	Positive	25.85
18.4	*A. aegypti* pupa	1	Positive	25.94
19.4	*A. aegypti* pupa	1	Positive	25.92
20.4	*A. aegypti* pupa	1	Positive	26.48
21.4	*Culex* larva	1	Negative	
22.4	*Culex* larva	1	Negative	
23.4	*Culex* larva	1	Negative	
24.4	*Culex* larva	1	Negative	
25.4	*Culex* pupa	1	Negative	
26.4	*Culex* pupa	1	Negative	
27.4	*A. aegypti* larva/*Culex* larvae	1/12	Positive	33.00
28.4	*A. aegypti* larva/*Culex* larvae	1/12	Positive	38.21
29.4	*A. aegypti* larva/*Culex* larvae	1/12	Positive	30.60
30.4	*A. aegypti* larva/*Culex* larvae	1/12	Positive	31.35

[a] *n* = number of specimens.

TABLE V

FIELD EVALUATION OF *Aedes aegypti* PCR ASSAY SENSITIVITY AND SPECIFICITY

Sample	Sample Composition: Blind Panel	n^a	PCR Results	Cycles (C_t)
1.5	*A. aegypti*	1	Positive	20.71
2.5	*A. aegypti*	1	Positive	24.24
3.5	*A. aegypti* larva	1	Positive	28.32
4.5	*A. aegypti* larva	1	Positive	26.91
5.5	*A. aegypti* larva	1	Positive	28.19
6.5	*Culex* larva	1	Negative	
7.5	*Culex* larva	1	Negative	
8.5	*Culex* larva	1	Positive	39.03
9.5	*A. aegypti* larva/*Culex* larvae	1/3	Positive	30.75
10.5	Pupa unknown (presumed *Culex*)	1	Negative	
11.5	Pupa unknown (presumed *Culex*)	1	Negative	
12.5	*A. aegypti* larva/*Culex* larvae with debris	1/12	Positive	33.81
13.5	*A. aegypti* larva/*Culex* larvae with debris	1/12	Negative	
14.5	*A. aegypti* larva/*Culex* larvae with debris	1/12	Positive	38.60
15.5	*A. aegypti* larvae with debris	12	Positive	30.81
16.5	*Culex* larvae with debris	12	Negative	
A.5	Debris only, *Culex* container	0	Negative	
B.5	Debris only, *A. aegypti* container	0	Negative	

[a]n = number of specimens.

egg pool sizes of five to 10 exceed surveillance requirements; therefore, assay sensitivity was not optimized to a limit of detection of a single egg. Inhibition of PCR did not occur with *A. aegypti*-spiked pools of non-*A. aegypti* species (Tables I and II).

Field Evaluations

Field testing of the assay with a known panel of *A. aegypti* and *Culex* spp., consisting of individual and mixed pools (n = 30) of adult mosquitoes, pupae, and larvae, demonstrated 100% in vitro sensitivity (22 of 22 samples) and 97% specificity (29 of 30 samples), with one false-positive result (Table IV). A single female *Culex* appeared to test positive after 40 PCR cycles (C_t, 40.52). Field testing of an *A. aegypti* and *Culex* spp. blind panel (n = 16), consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae, demonstrated 90% concordance in sensitivity (nine of 10 samples) and 88% concordance in specificity (14 of 16 samples) (Table V). One *A. aegypti* in a pool of 12 *Culex* produced a negative result, and a single *Culex* larva registered as a false-positive result.

Discussion

The *A. aegypti* real-time PCR assay described in this work clearly shows that both adult and immature specimens of this species can be accurately and rapidly identified by using the R.A.P.I.D., from both pure-culture and mixed-species pools. In laboratory and field-based testing, in vitro sensitivity and specificity results were concordant. In regard to the limit of detection in large mosquito pools, pools of >100 mosquitoes are not technically practical with our current method of nucleic acid extraction; therefore, pools that exceeded 100 mosquitoes were not evaluated in this study. Egg pool sizes of five to 10 exceed surveillance requirements; therefore, assay sensitivity was not optimized to a limit of detection of a single egg. In laboratory testing, a single female *Culex* appeared positive after 40 PCR cycles, and a field-collected *Culex* larva also reported late as a positive result. These data represent very weak fluorescence and are likely attributable to cross-contamination, because these specimens might have picked up some *A. aegypti* tissue when in combined storage before separation. These data are not indicative of a failure in assay specificity. Although a mosquito pool composed of a single *A. aegypti* and 12 *Culex* produced no detectable fluorescence, overall in vitro sensitivity results met an appropriate level of confidence for continued testing as a potential method in vector surveillance.

These preliminary data show promise in the field utility and practicality of a rapid and accurate, genomics-based, vector identification system. This method may offer a faster and more direct approach to identifying container-breeding *Aedes* species, by eliminating the time-consuming requirements of rearing adults from eggs collected in ovitraps. However, we have not yet fully evaluated the specificity of our assay with other mosquito taxa and, until such data are obtained, we consider these data preliminary. Validation testing of assay specificity will remain an ongoing process as additional species of *Aedes* (*Stegomyia*) and other mosquito taxa become a part of our continually expanding nucleic acid archive.

PCR-based genetic assays may ultimately offer a powerful tool for conducting surveillance of important vector species without the requirement of basing identification on adult stages. Identifying mosquitoes can prove challenging for untrained observers, even with simplified diagnostic information.[23] We think that our findings may have application for mosquito researchers and public health organizations requiring rapid identification of large numbers of samples or diverse samples that may contain multiple vector species, rather than using traditional, time-consuming, sorting and identification methods. The U.S. Air Force offers formal training on the R.A.P.I.D. system for candidacy for a biological augmentation team. A biological augmentation team consists of two members, officer and enlisted, qualified to deploy and operate the system in disease agent surveillance. Currently, training emphasis is on biowarfare agent surveillance; however, vector-borne disease agent surveillance curricula are planned.

Our assay system allows rapid field identification of adult,

larval, pupal, and egg stages of *A. aegypti*. We are in the process of expanding detection capability to include additional vectors and pathogens of military importance. Dengue virus universal and serotype 1 to 4 real-time PCR assays have been completed,[24] and *Phlebotomine* and *Leishmania* species assays are in development.

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